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Peanut polyamines may be non-allergenic†

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Abstract: Polyamines such as putrescine, spermidine and spermine have been implicated in preventing food allergies in early life, but they have also been reported to be able to bind to immunoglobulin E (IgE) antibodies *in vitro* (ie they are possibly allergenic). The objective of this study was to determine if polyamines bind *in vitro* to IgE antibodies from a pooled serum of five peanut-allergic individuals. Levels of polyamines were also determined by ion-exchange chromatography. Indirect and inhibition enzyme immunosorbent assays (ELISAs) were used to determine the IgE binding or allergenic properties of polyamines. Results showed that, of the three polyamines, spermidine was predominant in peanuts. In both indirect and inhibition ELISAs, IgE antibodies did not bind to the polyamines. It was concluded that polyamines from peanuts, unlike peanut proteins, are not allergenic or an additional threat to patients who are allergic to peanuts.
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INTRODUCTION

Polyamines such as putrescine [$H_2N(CH_2)_4NH_2$], spermidine [$H_2N(CH_2)_4NH(CH_2)_3NH_2$] and spermine [$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$] are nitrogen-containing polycationic compounds widely distributed in plants, microorganisms, and animal tissues.[1,2] Like other growth factors, polyamines are involved in nearly every step of DNA, RNA and protein synthesis, and are thus essential for cell proliferation and growth.[3,4] Polyamines provided by food have a potential role in the development of the digestive system, and have been related to the intestinal integrity and permeability to macromolecules in breast milk-fed infants.[5–9] One study has shown that the probability of developing food allergy can reach 80% if the polyamine concentration in the milk is low.[6] Also, insufficient intake of polyamines may result in the induction of sensitization to dietary allergens.[6,7] All these observations have implicated polyamines in preventing food allergies in early life. A protective effect of polyamines against alimentary allergies has been suggested.[8,9] Despite the benefits, polyamines have also been reported to bind to human immunoglobulin E (IgE) antibodies *in vitro*.[10] This observation suggests that polyamines may be allergenic, and is contradictory to the previous findings that polyamines may be beneficial in early life.[5–9]

In recent years, peanut allergy has received a lot of attention. Most of the attention and research have focused on the proteins Ara h 1, Ara h 2 and Ara h 3, which are major peanut allergens.[11] The role of other peanut components in allergenicity has not been well defined. Recently, we have reported that Maillard reaction adducts are one of the peanut components that may be responsible for the increased IgE binding or allergenicity in peanuts during roasting.[12–16] In view of the finding that polyamines may bind to IgE,[10] we were interested in determining whether polyamines are also components that may play a role in peanut allergenicity. In this study, the objective was to determine whether polyamines from peanuts are allergenic. High-performance liquid chromatography (HPLC) was also performed to determine which polyamine is predominant in peanuts.

MATERIALS AND METHODS

Chemicals

Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, 1,7-diaminoheptane, rabbit anti-human IgE-peroxidase conjugate, *o*-phenylenediamine, Tween 20 and Tris buffer saline (TBS) were obtained from Sigma Chemical Co (St Louis, MO, USA). SuperBlock solution, bicinchoninic acid (BCA)-protein assay kit, and *o*-phthalaldehyde (OPA) were purchased from Pierce Chemicals Co (Rockford, IL, USA). Perchloric acid was purchased from Fisher Scientific (Houston, TX, USA). Sera from five individuals with peanut allergy were obtained from

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the University of Arkansas, Children's Hospital (Little Rock, AR, USA). Peanuts (*Arachis hypogaea* L var Florunner) were obtained from the USDA-ARS National Peanut Research Laboratory (Dawson, GA, USA).

Preparation of peanut polyamine extracts and HPLC analyses

Preparation of extracts and chromatographic separation of polyamines were performed according to the method of Ohta *et al*.[17] with some modifications. Extract of polyamines was prepared by stirring 1 g of defatted peanut meal for 18 h at 4 °C in 7 ml of 5% perchloric acid ($HClO_4$) containing an internal standard 1,7-diaminoheptane (5 μg ml^{-1}). This was followed by centrifuging the mixture at 7000 × *g* for 20 min. The resultant supernatant or polyamine extract was neutralized and concentrated using a Savant Speed Vac Evaporator. For polyamine analyses, a high-performance liquid chromatograph (HPLC, Dionex) equipped with a cation exchange CS2 column (4 × 250 mm, Dionex) and a Dionex FDM fluorescence detector was used. Polyamines (20 μl of a concentrated extract) were separated on the CS2 column using a gradient elution program consisting of a mobile phase A and B, where phase A was 0.1 M citrate buffer, pH 5.6 containing 2 M KCl and 13.4 mM EDTA, and phase B was deionized water. The flow rate was 2 ml min^{-1}. The column eluate was then reacted with an OPA reagent containing 0.1% OPA, 0.3% 2-mercaptoethanol and 3% methanol at a flow rate of 0.6 ml min^{-1}. Fluorescence detection was monitored at an excitation wavelength of 356 nm and an emission wavelength of 450 nm. Quantification was accomplished in duplicate by calculating the integrated areas of sample peaks against the standard peak, 1,7-diaminoheptane. The percent recoveries for standard, putrescine, spermidine, and spermine were 96.60, 97.54, 101.54 and 109.98, respectively. Values are means of three replicate experiments. Statistical analyses, using Student's *t*-test at 95% confidence, were performed to determine the difference in levels between the polyamines.

Preparation of peanut allergen extracts

Extracts from defatted peanut meals were prepared as previously described.[18] Briefly, defatted peanut meals (40 mg) were stirred in 0.3 ml of 0.02 M sodium phosphate, pH 7.4, for 30 min at 4 °C, followed by centrifugation at 8500 × *g* for 10 min. The resultant supernatants or allergen extracts were used for enzyme immunosorbent assays (ELISA). Concentration of proteins in the extract was determined using the BCA assay.

Preparation of polyamine- and allergen-coated plates for ELISA

Polyamine-coated plates were prepared, according to the method of Delcros *et al*.,[19] by covalently attaching the polyamines to gelatin coated onto the plates. Briefly, gelatin-coated plates were treated with 0.5% glutaraldehyde in 50 mM borate buffer, pH 8, for 30 min at room temperature. After three washes with deionized water, 100 μl of a polyamine solution (10 mM) or a peanut polyamine extract in 50 mM borate, pH 8, 15 mM sodium cyanoborohydride, was added to each well. Plates were then incubated overnight at room temperature, followed by four washes with Tris buffer saline (TBS)/Tween 20. Allergen-coated plates were prepared according to the method of Chung *et al*.[18] by incubating plates with a peanut allergen extract (10 μg ml^{-1}) at 37 °C for 2 h. All of the coated plates were blocked with SuperBlock (1:1) in TBS/Tween 20.

Determination of IgE binding of polyamines and allergens in ELISA

A pooled serum (100 μl) containing IgE antibodies (1:20) of five peanut-allergic individuals was added to the polyamine- or allergen-coated plate, and incubated at room temperature for 45 min. Plates were then washed four times with TBS/Tween 20, and incubated with a rabbit anti-human IgE peroxidase conjugate (1:500; 100 μl) for 15 min at room temperature. Plates were again washed, and incubated with a substrate solution (100 μl) containing *o*-phenylenediamine (0.5 mg ml^{-1}), 0.03% hydrogen peroxide in 0.1 M citrate buffer, pH 5.5 for 15 min at 37 °C. The reaction was stopped with 4 M sulfuric acid (50 μl). Absorbance was read at 490 nm with a CERES 900C plate reader (Bio-Tek Instruments Inc, Winooski, VT, USA). Values are mean of three replicate experiments.

In addition, a competitive inhibition ELISA was carried out according to the method of Chung *et al*.[18] Briefly, polyamines or allergens from different peanut extracts (50 μl) at various concentrations (1–1000 μg ml^{-1}) were mixed with a pooled serum containing IgE antibodies (1:20; 50 μl), and were then added to an allergen-coated plate. After incubation for 45 min at room temperature, the plate was washed four times with TBS/Tween 20. A rabbit anti-human IgE peroxidase conjugate was added as described above, followed by washes and incubation with the substrate solution. In Fig 4, the absorbance value of a sample containing IgE antibodies and the polyamine or protein extract is represented by B, while B_o represents the absorbance value of a control containing IgE only. Values are represented as means of three replicate experiments.

RESULTS AND DISCUSSIONS

Polyamines in peanuts

In this study, polyamines were extracted from raw peanuts rather than the roasted. This is because roasted peanuts may have very low levels of polyamines due to heating,[20,21] thus requiring vigorous concentration steps to prepare the extracts, and, as a result, matrix effects may exist and interfere with HPLC

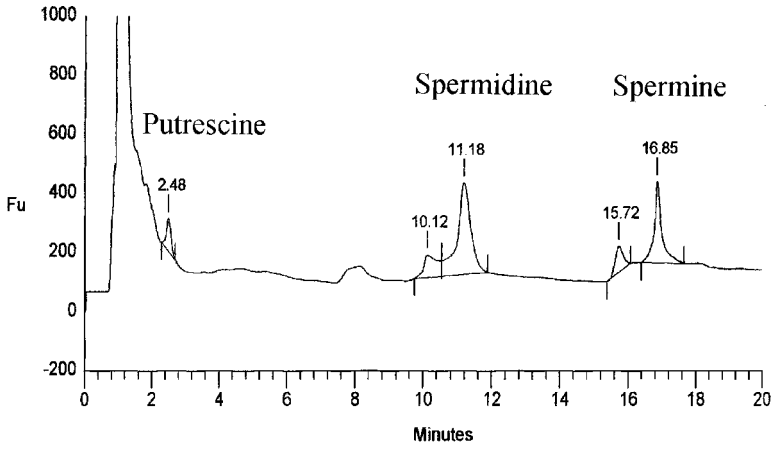

Figure 1. Separation of peanut polyamines by HPLC. Polyamines were separated on a cation exchange CS2 column (4 × 250 mm) using a gradient elution program (phase A = 0.1 M citrate buffer, pH 5.6 containing 2 M KCl and 13.4 mM EDTA; phase B = deionized water). Detection was achieved using an OPA fluorescent reagent. Standard 1,7-diamineheptane is at 10.12 min.

and ELISA assays. A typical separation of peanut polyamines by cation exchange chromatography is shown in Fig 1. Putrescine was eluted first from the column, followed by spermidine and spermine. The retention times for putrescine, spermidine, and spermine were 2.48, 11.18 and 16.85 min, respectively. The standard, 1,7-diaminoheptane, was at 10.12 min. Peanuts usually undergo a curing or drying process before roasting. Analyses of peanuts obtained before and after curing indicate that levels of spermidine increased after curing while putrescine and spermine decreased (Fig 2). This pattern is similar to that in soybeans[22] and is believed to be the result of various enzyme reactions. Among these reactions, the conversion of putrescine to spermidine by the enzyme spermidine synthase is considered predominant.[1] Following this reaction, spermidine can further be converted to spermine by another enzyme, spermine synthetase. As a result, spermine may increase. However, in this study, spermine was seen to decrease rather than increase after curing (Fig 2). This decrease in spermine may be due to osmotic stress occurring during curing.[23] Several studies have indicated that polyamines can increase or decrease in response to environmental or physiological stress, and thus protect plants from stress.[20,24] In osmotic stress, spermidine is reported to increase and confer stress tolerance. This agrees with our data, which show that spermidine increased during curing, and supports our postulation that osmotic stress may be responsible for changes of polyamines in peanut curing. Overall, in peanuts the highest level is spermidine ($92 \pm 2.2\,\mu g\,g^{-1}$ dry defatted meal), followed by spermine ($21 \pm 0.5\,\mu g\,g^{-1}$) and putrescine ($4 \pm 0.1\,\mu g\,g^{-1}$).

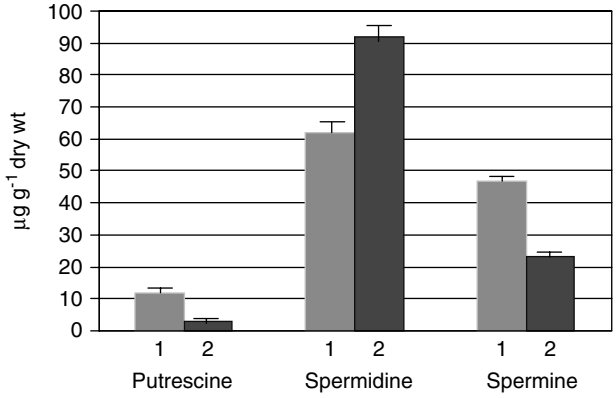

Figure 2. Levels of polyamines before (1) and after (2) peanut curing. Polyamines were determined as described in Fig 1 against the peak area of the standard 1,7-diamineheptane. Values are mean ± SD of three separate determinations. Values of the three polyamines (before or after curing) were significantly different from each other at 95% confidence (Student's *t*-test).

IgE binding or allergenic properties of polyamines

Indirect and competitive inhibition ELISAs were used to determine IgE binding to polyamines. In the indirect ELISA, individual polyamines (putrescine, spermidine or spermine) or polyamines from an extract of cured peanuts were immobilized through covalent linkage to a gelatin-coated plate, and then allowed to react with the IgE antibodies. This method has proven effective despite one of the primary amino groups (from polyamine) being removed during immobilization.[19] A number of studies have also had success in determining IgE binding, using similar methods (ie attachment of small molecules via their functional groups to proteins).[25–27] For comparison with the polyamine–gelatin-coated plate, IgE antibodies were also incubated with a plate coated with peanut allergens. Results showed that there was no binding between IgE and polyamines, but allergens coated to the plate (Fig 3). Similar results were obtained when individual serum rather than a pooled serum was used (data not shown).

In the competitive inhibition ELISA, free polyamines instead of immobilized ones were allowed to react with IgE antibodies before transferring to a peanut allergen-coated plate. A flat curve was obtained (Fig 4), indicating that IgE antibodies or their bindings to the allergen-coated plate were not inhibited by

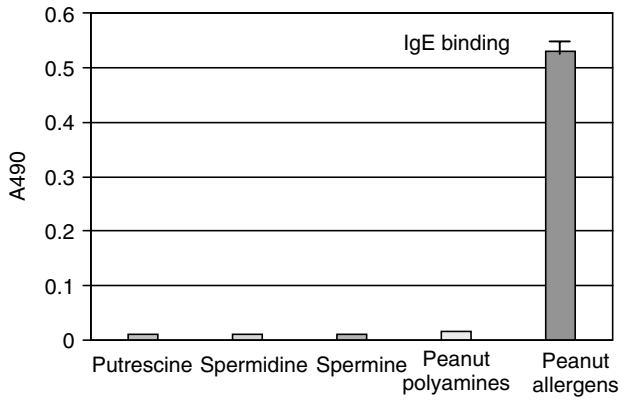

Figure 3. IgE binding of peanut polyamines and allergens in indirect ELISA. Polyamines as indicated were immobilized onto gelatin-coated plates and allowed to react with IgE antibodies (1:20) from a pooled serum of five peanut-allergic individuals. A rabbit anti-human IgE-peroxidase conjugate (1:500) and a substrate solution of *o*-phenylenediamine were used to detect IgE binding. Bindings of IgE to polyamine-coated and allergen-coated plates were compared. Values are means ± SD of three separate determinations.

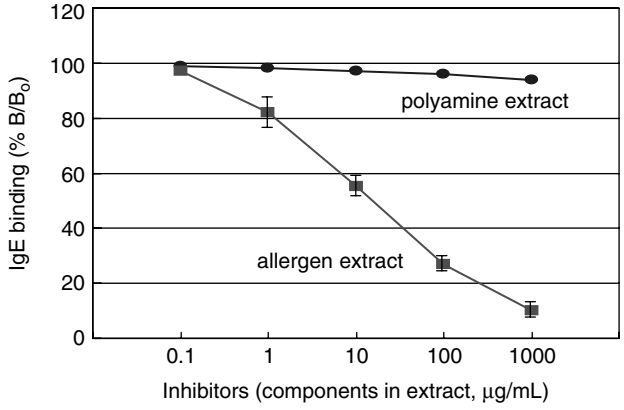

Figure 4. Competitive inhibition ELISA of IgE binding to peanut polyamines and allergens. Polyamines or allergens from extracts of cured peanuts at the concentrations indicated were mixed with IgE antibodies (1:20) from a pooled serum of five peanut-allergic individuals before transferring to an allergen-coated plate. Inhibition of IgE binding to the plate was detected using the same reagents as described in Fig 3. Values are means ± SD of three separate determinations.

peanut polyamines or individual polyamines (not shown). In contrast, a reduction in IgE binding to the plate was observed when free peanut allergens (from extracts), which competed with the bound allergens for binding to IgE, were used instead of free polyamines. All this indicates that IgE antibodies from peanut-allergic individuals do not bind to polyamines (putrescine, spermidine or spermine).

It should be noted that the above finding was an *in vitro* study. The *in vivo* role of polyamines in peanut allergenicity still remains unclear. It has long been suggested that, to prevent peanut allergies, infants and young children should avoid peanut products until they are 3 years old. However, there is little research to support that theory. In this study, we demonstrated that peanuts have a high level of spermidine. This compound, along with others, has been implicated in the prevention of food allergies in early life.[5–9] If this is true and intake of polyamines is sufficient, eating peanuts at an early age may not be as inappropriate as previously thought.

CONCLUSIONS

The objective of this study was to determine the allergenicity of putrescine, spermidine and spermine from peanuts. HPLC analyses indicated that spermidine was predominant among the three polyamines. In contrast to the study of Zhao *et al*.,[10] our data showed that none of the polyamines bound or inhibited IgE in ELISAs. This suggests that polyamines from peanuts may not be allergenic. Also, this implies that peanuts may be good for children of very young age, in view of the fact that peanuts contain predominantly spermidine, which is reported to have a role in protection against food allergies in early life.[5–9]

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